consisting of a thermocycler, a multichannel pipettor, a sample handler, a plate handler, a gel loading system, an automated transformation system, a gene sequencer, a colony picker, a bead picker, a cell sorter, an incubator, a light microscope, a fluorescence microscope, a spectrofluorometer, a spectrophotometer, a luminometer a CCD camera and combinations thereof.

Please replace the paragraph beginning at page 7, line 24, with the following rewritten paragraph:

The methods of the invention can be briefly described as follows. Gene cloning comprising the rapid isolation of cDNA clones is facilitated by taking advantage of the catalytic function of the RecA enzyme, an essential component of the E. coli DNA recombination system, which promotes formation of multi-stranded hybrids between ssDNA probes and homologous double-stranded DNA molecules. The targeting of RecA-coated ssDNAs to homologous sequences at any position in a duplex DNA molecule can produce stable D-loop hybrids. The probe strands in the D-loop are stable enough to be manipulated by conventional molecular biology procedures. The stability of these deproteinized multi-stranded hybrid molecules at any position in duplex molecules allows the application of D-loop methods to many different dsDNA substrates, including duplex DNA from cDNA, genomic DNA, or YAC, BAC or PAC libraries. Recombinase coated biotinylated-probes are targeted to homologous DNA molecules and the probe:target hybrids are selectively captured on streptavidin-coated magnetic beads. The enriched plasmid population is eluted from the beads, precipitated, resuspended, and used to transform bacteria or the cells. The resulting colonies are screened by PCR and colony hybridization to identify the desired clones. Using this method over 100,000 fold enrichment of the desired clones can be achieved. Furthermore, once the target sequence is cloned, large numbers of variants can be easily generated, again using EHR techniques. These variants can be screened in a wide variety of phenotypic screens, either in the presence or absence of drug candidates.

Please replace the paragraph beginning at page 8, line 14, with the following rewritten paragraph:

The methods of the invention are directed to the cloning of target nucleic acid sequences. By "target nucleic acid sequence" or "predetermined endogenous DNA sequence" and "predetermined target sequence" we refer to polynucleotide sequences contained in a target cell and DNA libraries. Such sequences include, for example, chromosomal sequences (e.g., structural genes, regulatory sequences including promoters and enhancers, recombinatorial hotspots, repeat sequences, integrated proviral sequences, hairpins, palindromes), episomal or extrachromosomal sequences (e.g., replicable plasmids or viral replication intermediates) including chloroplast and mitochondrial DNA sequences.

Please replace the paragraph beginning at page 10, line 15, with the following rewritten paragraph:

A metabolically-active cell is a cell, comprising an intact nucleoid or nucleus, which, when provided nutrients and incubated in an appropriate medium carries out DNA and RNA synthesis for extended periods (e.g., at least 12-24 hours). Such metabolically-active cells are typically undifferentiated or differentiated cells capable or incapable of further cell division (although non-dividing cells many undergo nuclear division and chromosomal replication), although stem cells and progenitor cells are also metabolically-active cells.

Please replace the paragraph beginning at page 10, line 22, with the following rewritten paragraph:

In some embodiments, the target sequence is a disease allele. As used herein, the term "disease allele" refers to an allele of a gene which is capable of producing a recognizable disease. A disease allele may be dominant or recessive and may produce disease directly or when present in combination with a specific genetic background or pre-existing pathological condition. A disease allele may be present in the gene pool or may be generated de novo in an individual by somatic mutation. For example and not limitation, disease alleles include: activated oncogenes, a sickle cell anemia allele, a Tay-Sachs allele, a cystic fibrosis allele, a Lesch-Nyhan allele, a retinoblastoma-susceptibility allele, a Fabry's disease allele, and a Huntington's chorea allele. As used herein, a disease allele encompasses both alleles associated with human diseases and alleles associated with recognized veterinary diseases. For example, the ΔF508 CFTR allele is a human disease allele which is associated with cystic fibrosis in North Americans.

Please replace the paragraph beginning at page 15, line 18, with the following rewritten paragraph:

In a preferred embodiment, two substantially complementary single-stranded targeting polynucleotides are used. The two complementary single-stranded targeting polynucleotides are usually of equal length, although this is not required. However, as noted below, the stability of the four strand hybrids of the invention is putatively related, in part, to the lack of significant unhybridized single-stranded nucleic acid, and thus significant unpaired sequences are not preferred. Furthermore, as noted above, the complementarity between the two targeting polynucleotides need not be perfect. The two complementary single-stranded targeting polynucleotides are simultaneously or contemporaneously introduced into a target cell harboring a predetermined endogenous target sequence, generally with at lease one recombinase protein (e.g., recA). Under most circumstances, it is preferred that the targeting polynucleotides are incubated with recA or other recombinase prior to introduction into a target cell, so

that the recombinase protein(s) may be "loaded" onto the targeting polynucleotide(s), to coat the nucleic acid, as is described below. Incubation conditions for such recombinase loading are described infra, and also in U.S.S.N. 07/755,462, filed 4 September 1991; U.S.S.N. 07/910,791, filed 9 July 1992; and U.S.S.N. 07/520,321, filed 7 May 1990, each of which is incorporated herein by reference. A targeting polynucleotide may contain a sequence that enhances the loading process of a recombinase, for example a recA loading sequence is the recombinogenic nucleation sequence poly[d(A-C)], and its complement, poly[d(G-T)]. The duplex sequence poly[d(A-C)•d(G-T)]n, where n is from 5 to 25, is a middle repetitive element in target DNA.

Please replace the paragraph beginning at page 19, line 32, with the following rewritten paragraph:

In a preferred embodiment, the gene family is involved in DNA mismatch repair, such as mutL, hexB and PMS1. Members of this family include, but are not limited to, MLH1, PMS1, PMS2, HexB and MulL. The protein consensus sequence is G-F-R-G-E-A-L (SEQ ID NO:1).

Please replace the paragraph beginning at page 20, line 1, with the following rewritten paragraph:

In a preferred embodiment, the gene family is the recA family. The bacterial recA is essential for homologous recombination and recombinatorial repair of DNA damage. RecA has many activities, including the formation of nucleoprotein filaments, binding to single stranded and double stranded DNA, binding and hydrolyzing ATP, recombinase activity and interaction with lexA causing lexA activation and autocatalytic cleavage. RecA family members include those from E. coli, Drosophila, human, lily, etc. specifically including but not limited to, E. coli recA, Rec1, Rec2, Rad51, Rad51B, Rad51C, Rad51D, Rad51E, XRCC2 and DMC1.

Please replace the paragraph beginning at page 29, line 8, with the following rewritten paragraph. Support is found at page 29, line 9 and page 12, lines 32-35.

In a preferred embodiment, the compositions find use in the cloning of target nucleic acids. In this embodiment, the EHR compositions are contacted with a nucleic acid library such as a cDNA library, genomic DNA, or YAC, BAC or PAC libraries. In general, any library that serves as a source of target sequences can be used. In addition, the target can be genomic DNA, cDNA, RNA, DNA plasmid or a combination of deoxyribo- and rebonucleotides. In addition, any target cells outlined herein may be used to generate a cDNA library for use in the invention. Furthermore, while not preferred in some embodiments, the nucleic acid library may actually be a library of target cells.

Please replace the paragraph beginning at page 34, line 11, with the following rewritten paragraph:

Fifty nanograms of each of the biotinylated probes are distributed to each well of the 96 well non-cross contamination (NCC) microplates and the sample volume in each well is brought up to 13 µl with H_20. All reactions are generally performed in duplicate or triplicate. The microplate is placed on the P2 position of the MWG-Biotech RoboAmp 4200 Robot deck.

Please replace the paragraph beginning at page 34, line 26, with the following rewritten paragraph:

In a preferred embodiment, the targeting polynucleotides are coated with RecA recombination protein to form nucleoprotein filaments. For each reaction, 6 µl of the 5X coating buffer (50 mM Tris-acetate, pH 7.5, 250 mM sodium-acetate, 10 mM Mg-Acetate, and 5 mM DTT), 3.7 ul of 16.2 mM ATPγS (Boeringer Mannheim), and 0.7 ul 1 mg/ml RecA (Promega) protein is combined in a 0.5 ul microfuge tube and placed in the 4°C cooled Position 1 of the reagent rack on the robot deck. The automated pipetter aspirates 10.4 ul of the coating mixture, the robotic lid handler uncaps each lid of the wells of the destination microplate (P5 position), and the pipettor dispenses the coating mix into the well with the denatured probe. The samples are optionally mixed by pipetting. After addition of the coating mix to each of the wells, the plate handler transfers the microplate to the themocycler and the samples are incubated at 37°C for 15 minutes to allow the recombinase to bind to the nucleic acid probes.

Please replace the paragraph beginning at page 34, line 37 and continuing to page 35, with the following rewritten paragraph:

In a preferred embodiment, the RecA-ssDNA nucleoprotein filaments are targeted to the desired cDNA clones. For each DNA library, 5 mg of library in a volume of 5 ul (adjusted to 5 ul with TE' if the library is at a stock concentration greater than 1 mg/ml) is mixed with 1.2 ul of 200 mM Mg-Acetate (final Mg concentration is 10 mM in targeting reaction in each well of the microplate at position P1 on the robot deck. Five microliters of the library mix is aspirated by the robotic liquid pipetter, the robotic lid handler uncaps the lid of the destination microplate (P5 position), and the pipetter dispenses the coating mix into the well with the nucleoprotein filaments. The samples are optionally mixed by pipetting. After addition of the coating mix to all of the wells, the plate handler transfers the microplate to the thermocycler and the samples are incubated at 37°C for 20 minutes to allow the hybridization of nucleoprotein filaments to homologous target nucleic acid. After hybridization, the microplate is transferred to the P5 position by the plate handler. From position 2 of the reagent rack, the robotic liquid pipetter aspirates and dispenses 1 µl of 50µg/ml salmon sperm competitor DNA into each well of

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the destination microplate (P5 position) and the samples are optionally mixed by pipetting. The microplate is transferred to the thermocycler and incubated for 5 minutes at 37°C. The microplate is then transferred to the P5 position on the robot deck.

Please replace the paragraph beginning at page 35, line 14, with the following rewritten paragraph:

a14

In a preferred embodiment, the targeted hybrid DNAs are deproteinized. The targeting of RecA coated ssDNA to homologous sequences at any position in a duplex DNA molecule produces stable D-loop hybrids after protein removal. For each reaction, 0.6 µl of the SDS solution (10 mg/ml) and 0.4 µl of Proteinase K (Boehringer Mannheim) is combined in a 1.5 ml microfuge tube and placed in position 3 of the reagent rack. The liquid pipetter aspirates and dispenses 1 µl of the SDS mixture into each well of the sample microplate and optionally mixes the samples by pipetting. The microplate is transferred to the thermocycler and incubated for 10 minutes at 37°C. The microplate is transferred to the P5 position and the liquid pipetter adds 1 µl of phenylmethyl-sulfonyl fluoride (PMSF) protease inhibitor (Boehringer Mannheim) from Position 4 of the reagent rack.

Please replace the paragraph beginning at page 35, line 31, with the following rewritten paragraph:

a15

The desired target sequences, usually cDNA, are then isolated. The non-homologous, unbound DNA is manually aspirated from each well of the microplate. Each well is washed three times with Wash buffer (10 mM Tris-HCl pH 7.5, 2 M NaCl, and 1 mM EDTA), incubated once with ddH_20 for 5 minutes at 37°C, and eluted with Elution Buffer (100 mM NaOH, 1mM EDTA). The DNA is transferred to a 1.5 ml microfuge tube precipitated with the addition of Precipitation Mix (2.75 M NaAcetate pH 7, 1.67 mg/ml Glycogen) and 500 µl of 100% ethanol. The samples are incubated at -70°C for 20 minutes or -20°C for 30 minutes and centrifuged for 20 minutes at 4°C. The pellets are washed once with 70% ethanol and air dried. The pellets are resuspended in TE'.

Please replace the paragraph beginning at page 36, line 1, with the following rewritten paragraph:

a16

In a preferred embodiment, the target nucleic acid is amplified in bacteria. The captured DNA (2 ul) is electroporated into DH5α competent cells (40 µl) using the BTX Electro Cell Manipulator 600 and the cells are shaken for 1 hour at 37°C. The cells are plated onto four LB-ampicillin plates or used to inoculate 100 ml LB-ampicillin and are grown overnight at 37°C. The cells are harvested from the plates or from the liquid cultures and the DNA is purified using Qiagen Plasmid Midi Kits (Qiagen) or the Toyobo DNA purification robot. This DNA is screened by PCR to verify the presence of the desired cDNA and then used in a second round of cloning reactions. Alternatively, the colonies from

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the plates are transferred to Hybond filters (Amersham-Pharmacia) and are screened by colony hybridization to a biotinylated or radiolabeled DNA probe and by PCR to identify the desired clones.

Please replace the paragraph beginning at page 43, line 3, with the following rewritten paragraph:

Thus, this embodiment proceeds as follows. A pool of targeting polynucleotides is made, each containing one or more mismatches. The probes are coated with recombinase as generally described herein, and introduced to the target sequence as outlined herein. Upon binding of the probes to form D-loops, homologous recombination can occur, producing altered target sequences. These altered target sequences can then be introduced into cells to produce target protein which can then be tested for biological activity, based on the identification of the target sequence. Depending on the results, the altered target sequence can be used as the starting target sequence in reiterative rounds of homologous recombination, generally using the same library. Preferred embodiments utilize at least two rounds of homologous recombination, with at least 5 rounds being preferred and at least 10 rounds being particularly preferred. Again, the number of reiterative rounds that are performed will depend on the desired end-point, the resistance or susceptibility of the protein to mutation, the number of mismatches in each probe, etc.

Q17

Please replace the paragraph beginning at page 43, line 24, with the following rewritten paragraph:

Similarly, in some embodiments, for some screens, preferred eukaryotic cells are embryonic stem cells (ES cells) and fertilized zygotes are preferred. In a preferred embodiment, embryonal stem cells are used. Murine ES cells, such as AB-1 line grown on mitotically inactive SNL76/7 cell feeder layers (McMahon and Bradley, Cell 62: 1073-1085 (1990)) essentially as described (Robertson, E.J. (1987) in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E.J. Robertson, ed. (Oxford: IRL Press), p. 71-112) may be used for homologous gene targeting. Other suitable ES lines include, but are not limited to, the E14 line (Hooper et al. (1987) Nature 326: 292-295), the D3 line (Doetschman et al. (1985) J. Embryol. Exp. Morph. 87: 21-45), and the CCE line (Robertson et al. (1986) Nature 323: 445-448). The success of generating a mouse line from ES cells bearing a specific targeted mutation depends on the pluripotence of the ES cells (i.e., their ability, once injected into a host blastocyst, to participate in embryogenesis and contribute to the germ cells of the resulting animal).

Q18

Please replace the paragraph beginning at page 49, line 34, with the following rewritten paragraph:

Flow cytometry is used for individual capture of magnetic and other beads, particles, cells, and organisms.

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Please replace the paragraph beginning at page 50, line 5, with the following rewritten paragraph:

– EXAMPLE 2: High Throughput Semi-Automated Gene Cloning –

Please replace the paragraph beginning at page 50, line 16, with the following rewritten paragraph:

– Sample RecA-mediated cloning results are easily quantified by examining data from a control library. These libraries are made by mixing a defined ratio of two plasmids, pHPRT and pUC. The rare plasmid (pHPRT) contains a 530 bp region of the HPRT gene inserted into the β-galactosidase gene and the abundant plasmid pUC carries the β-galactosidase gene. The probe in all reactions is homologous to the HPRT region in the rare plasmid. The ratio of pHPRT:pUC was 1:10,000, which represents the frequency of an abundant gene in a cDNA library. –

Please replace the paragraph beginning at page 50, line 30, with the following rewritten paragraph:

– A 318 bp biotin-HPRT probe was coated with recombinase and targeted to the control library. Positive colonies were rapidly screened by visualization of white colonies carrying the pHPRT plasmid or blue colonies carrying the pUC plasmid when plated on the chromogenic substrate 5-bromo-4-chloro-indolyl-D- β -galactoside (X-gal). –

Please replace the paragraph beginning at page 50, line 35, with the following rewritten paragraph:

– Primers used to generate 318 bp biotinylated HPRT probe for clone isolations:

hExo3-2A 5' ATCACAGTTCACTCCAGCCTC 3' (SEQ ID NO:2)

h/m300B 5' TATAGCCCCCCTTGAGCACACAG 3' (SEQ ID NO:3) –

Please replace the paragraph beginning at page 51, line 9, with the following rewritten paragraph:

– Sequence of Rad51C probe:

GTGAGTTTCCCGCTGTCTCCAGCGGTGCGGGTGAAGCTGGTGTCTGCGGGGTTCCAGACTG
CTGAGGAACTCCTAGAGGTGAAACCCTCCGAGCTTAGCAAAGAAGTGGGGATATCTAAAG
CAGAAGCCTTAGAAACTCTGCAAATTATCAGAAGAGAATGTCTCACAAATAAACCAAGAT
ATGCTGGTACATCTGAGTCACACAAGAAGTGTACAGCACTGGAACTTCTTGAGCAGGAGC
ATACCCAGGGCTTCATAATCACCTTC (SEQ ID NO:4) –

Please replace the paragraph beginning at page 51, line 23, with the following rewritten paragraph:

– Primers used to generate 267 bp biotinylated human Rad51C probe for Rad51C cDNA clone isolations:

Rad51C-F59 5' GTG AGT TTC CCG CTG TCT CC 3' (SEQ ID NO:5)

Rad51C-R325 5' GAA GGT GAT TAT GAA GCC CTG G 3' (SEQ ID NO:6) –

Please replace the paragraph beginning at page 51, line 34, with the following rewritten paragraph:

– A. Mouse Actin Gene Family cDNA cloning using a Human β-Actin Probe –

Please replace the paragraph beginning at page 51, line 40, and continuing to page 52, with the following rewritten paragraph:

– Sequence of 510 base pair human beta actin probe used in RecA protein-mediated mouse cDNA isolation:

GACTACCTCATGAAGATCCTCACCGAGCGCGGCTACAGCTTCACCACCACGGCCGAGCGG
GAAATCGTGCGTGACATTAAGGAGAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAAGAG
ATGGCCACGGCTGCTTCCAGCTCCTCCCTGGAGAAGAGCTACGAGCTGCCTGACGGCCAG
GTCATCACCATTGGCAATGAGCGGTTCCGCTGCCCTGAGGCACTCTTCCAGCCTTCCTTCC
TGGGCATGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTCCATCAGAAGTGTGACGTG
GACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTCTGGCGGCACCACCATGTACCCTG
GCATTGCCGACAGGATGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCA
AGATCATTGCTCCTCCTGAGCGCAAGTACTCGTGTGGATCGGCGGCTCCATCCTGGCCTCG
CTGTCCACCTTCCAGCAGATGTGGAT (SEQ ID NO:7) –

Please replace the paragraph beginning at page 52, line 21, with the following rewritten paragraph:

– Primers used to synthesize the biotinylated human actin probe:

Actin1: 5' ACGGACTACCTCATGAAGATCC 3' (SEQ ID NO:8)

Actin2: 5' ATCCACATCTGCTGGAAGGTG 3' (SEQ ID NO:9) –

Please replace the paragraph beginning at page 52, line 25, with the following rewritten paragraph. Support for the changes is found at page 8, lines 11-12, page 50, lines 30-33, and page 51, line 35 to page 52, line 33.

– In the gene cloning procedure, biotin-labeled cssDNAs were denatured and coated with RecA recombinase protein. These nucleoprotein filaments were targeted to homologous target DNAs in a DNA library. The hybrids were deproteinized and captured on streptavidin-coated magnetic beads.

The homologous dsDNA target was eluted and transformed into bacteria. After recombinase-mediated targeting, clone capture, and DNA transformation into bacterial cells, the resulting colonies were screened by PCR, colony hybridization to filters, and DNA sequencing to identify the actin-related clones. Colony hybridization involved the transfer of the colonies from the plates to Hybond filters (Amersham), denaturation of the DNA, neutralization of the filters, and hybridization of a radiolabeled or biotinylated ssDNA probe to the positive clones to confirm that the clone isolation procedure was successful. The clones were randomly picked and cultured for DNA purification and sequencing. The use of recombinase-mediated homologous targeting has significant advantages over thermodynamically driven DNA hybridization such as PCR-based DNA amplification, which is widely used to isolate gene homologs and can have non-specific background hybridizations and artifacts due to improper renaturation of repeated sequences. –

Please replace the paragraph beginning at page 53, line 12, with the following rewritten paragraph:

– Sequence of human Rad51A biotinylated probe used to capture mouse Rad51A cDNA from mouse embryo cDNA library:
ATTGACACTGAGGGTACCTTTAGGCCAGAACGGCTGCTGGCAGTGGCTGAGAGGTATGGT
CTCTCTGGCAGTGATGTCCTGGATAATGTAGCATATGCTCGAGCGTTCAACACAGACCACC
AGACCCAGCTCCTTTATCAAGCATCAGCCATGATGGTAGAATCTAGGTATGCACTGCTTAT
TGTAGACAGTGCCACCGCCCTTTACAGAACAGACTACTCGGGTCGAGGTGAGCTTTCAGCC
AGGCAGATGCACTTGGCCAGGTTTCTGCGGATGCTTCTGCGACTCGCTGATGAGTTTGGTG
TAGCAGTGGTAATCACTAATCAGGTG (SEQ ID NO:10) –

Please replace the paragraph beginning at page 53, line 21, with the following rewritten paragraph:

– Primers used to synthesize 329 bp biotinylated human Rad51A probe:
Rad51A-F689 5' ATT GAC ACT GAG GGT ACC TTT AGG 3' (SEQ ID NO:11)
Rad51A-R1017 5' CAC CTG ATT AGT GAT TAC C 3' (SEQ ID NO:12) –

Please replace the paragraph beginning at page 53, line 25, with the following rewritten paragraph:

– After recombinase-mediated targeting, clone capture, and DNA transformation into bacterial cells, the resulting colonies were screened by PCR, colony hybridization to filters, and DNA sequencing to identify the Rad51A clones. Colony hybridization involved the transfer of the colonies from the plates to Hybond filters, denaturation of the DNA, neutralization of the filters, and hybridization of a radiolabeled or biotinylated ssDNA probe to the positive clones. The desired clones were picked and cultured for DNA purification and sequencing. The recombinase-mediated targeting and capture is a

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powerful method to isolate interspecies DNA clones. The mouse Rad51A cDNA was cloned using a probe containing the human Rad51A sequence in RecA protein-mediated targeting and capture reactions. –

Please replace the paragraph beginning at page 53, line 35, and continuing to page 54, with the following rewritten paragraph:

– Example 4: Gene cloning by amplification of DNA on solid matrices, e.g. beads, chips, plates
Rare or limited nucleic acids have been amplified by transformation of the captured DNA into bacterial cells. As an alternative to amplifying in biological hosts, nucleic acids can be immobilized onto beads, chips, plates, optical fibers, or other solid supports and can be cloned by PCR or other duplication methods to potentially generate 10^4-10^8 copies of each cDNA clone or genomic fragment. Multiple sequence variants (gene families, polymorphic genomic fragments, etc.) can be amplified in parallel on solid matrices and can be separated by fluorescent sorting methods, microarray matrices, etc. and can be sequenced. Differentially expressed genes can be compared within one library or the expression of particular genes can be compared between libraries. Gene cloning and amplification will allow the identification of rarely expressed genes and the elucidation of single-nucleotide polymorphisms (SNP)-bearing fragments that are differentially represented from two populations of individuals. Additional applications include gene amplification (cloning); mutagenesis, modifications (mutations, gene duplications, gene conversion, etc.), and evolution of genes; isolation of gene families, gene orthologs, and paralogs; differential gene expression; single and multiple nucleotide polymorphisms (genetic variation); genotyping and haplotyping; multigenic trait analysis and inference, allelic frequency; association of alleles; association of haplotypes with phenotypes (find trait-associated genes and trait associated polymorphisms); identification of disease-associated alleles and polymorphisms; linkage mapping and disequilibrium, loss of heterozygosity (LOH) and other chromosomal aberration diagnostics; single nucleotide polymorphism (SNP) validation; nucleic acid library production, subtraction and normalization; gene mapping; gene segregation analysis. –

C33

Please replace the paragraph beginning at page 54, line 28, with the following rewritten paragraph. Support is found at page 8, line 14 to page 9, line 6.

– The genomic DNA fragment encoding a desired differentially expressed gene can be isolated and cloned. Nucleic acids probes (oligonucleotides, PCR fragments) are first attached to solid matrices (beads, chips, filters, etc.), coated with recombinase protein, and are used to capture target cDNAs from libraries or any population of nucleic acid. The expression levels of the cDNAs will be determined in two or more populations (of cells, tissues, etc.). For example, to capture genomic DNA of a differentially expressed gene, the desired cDNA of an overexpressed or underexpressed gene that was captured on the solid matrix is coated with recombinase and is used as the probe to capture the genomic

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DNA fragment from a library (genomic, cell or tissue extract, etc.). The desired genomic DNA is amplified on the solid matrix or is first cleaved from the matrix and then amplified. –

Please replace the paragraph beginning at page 55, line 6, with the following rewritten paragraph:

a35

– The desired cDNA or genomic fragment or other nucleic acid can be isolated on solid supports as described above using recombinase-mediated gene targeting. The in vitro transcription of the cDNA or gene can be performed on the solid matrix. In addition, in vitro translation of the resulting mRNA to protein can be performed on the solid matrix. The protein products derived from in vitro transcription and translation can be used directly in compound and drug screening assays. –

Please replace the paragraph beginning at page 55, line 22, with the following rewritten paragraph:

a36

– To generate mutant substrates for high throughput phenotyping, exact or degenerate EHR probes are used to generate a library of transgenic cells or organisms with single or multigene knockouts, corrections, or insertion of single nucleotide polymorphisms (SNPs) in organisms (such as zebrafish and C.elegans), totipotent cells (such as embryonic stem [ES] cells), proliferative primary cells (such as keratinocytes or fibroblasts), and transformed cell lines (such as CHO, COS , MDCK, and 293 cells). ES cells can be further differentiated into embryoid bodies, primitive tissue aggregates of differentiated cell types of all germinal origins, and keratinocytes can be induced to stratify and differentiate into epidermal tissue. DNA is delivered to cells using standard methods including lipofection, electroporation, microinjection, etc. and mutagenized cells, tissues and organisms can be used for phenotypic and drug screening for validation of gene targets (see below). The high-throughput platform is designed to biovalidate gene targets by screening chemical or biological libraries that enhance or cause reversion of the phenotype. The high-throughput EHR phenotypic screening technology allows genetic profiling of compound libraries, selection of new drug leads, and identification and prioritization of new drug targets. –

Please replace the paragraph beginning at page 55, line 38, and continuing to page 56, with the following rewritten paragraph:

a37

– There are germline signals that act by modulating the activity of insulin/IGF-1 (insulin-like growth factor) pathway that are known to regulate the aging of C. elegans. It has been established that the insulin/IGF-1-receptor homologue, DAF-2, plays a role in signaling the animal's rate of aging since mutants with reduced activity of the protein have been shown to live twice as long as normal C. elegans. EHR is used to introduce additional mutations into DAF-2, and to identify and/or isolate additional DAF-2 family members using a degenerate HMT, consisting of a recombinase-coated complementary single-stranded DNA consensus sequence. This technology enables cloning of interspecific DAF-2 homologues, including

zebrafish, mouse, and human. EHR is used to disrupt DAF in zebrafish, and its effect on the aging process is assessed by screening for organisms with an extended lifespan. The same procedure is used to modify mouse or human DAF in primary cells, including keratinocytes and fibroblasts, and the proliferative capacity of cells is ascertained. Specific related genes are disrupted using EHR, and degenerate HMT probes are directly introduced into cells and animals to modify DAF-2-related genes, in order to analyze aberrant phenotypes. –

Please replace the paragraph beginning at page 56, line 13, with the following rewritten paragraph:

– EHR is also used to generate Green Florescent protein (GFP) DAF-2 wild-type (WT) and mutant chimeras, and the subcellular localization of the proteins are determined. The genes of interest are biovalidated by screening for drugs that enhance or cause revert of the altered phenotype. –

Please replace the paragraph beginning at page 56, line 29, and continuing to page 57, with the following rewritten paragraph:

– Gata5 is an essential regulator in controlling the growth, morphogenesis, and differentiation of the heart and endoderm in zebrafish. Gata5 is a master switch that induces embryonic stem cells to become heart cells. From loss- and gain-of function experiments, the zinc finger transcription factor Gata5 has been shown to be required for the production of normal numbers of developing myocardial precursors and the expression of normal levels of several myocardial genes in zebra fish. EHR is used to clone related Gata5 family members (zebrafish, mouse and human), and to introduce additional mutations in Gata5 and its homologues in zebrafish. EHR is used to ablate or modify Gata5 function in mouse embryonic stem (ES) cells, which are differentiated into embryoid bodies (EBs). ES cells are plated into duplicate wells to undergo differentiation into EBs, and one set is prescreened using immunofloresence with antibodies to terminally differentiated gene products to eliminate EBs which undergo normal differentiation. EBs defective in terminal differentiation are disaggregated, replated, and cell sorted to score for cardiac cell populations to determine the effect of the targeted mutation on the differentiation process. Gene expression profiles are determined using microarrays, DNA chips, or related technologies. Cultured mutant EBs are used for drug screening. Additionally, the same set of experiments can be repeated with human embryonic stem cells to determine if Gata5 plays a similar role in human tissue, and these and the mouse cultured mutant EBs can be used for drug screening. –

Please replace the paragraph beginning at page 57, line 9, with the following rewritten paragraph:

– Disruption of gene function from a single allele is adequate to cause a phenotype in cells for a subset of genes with tightly regulated abundance. In examples D-F, disruption of a single allele results in a screenable phenotype. Disruption of a single allele of either VEGF or GATA-1 in embryonic stem

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cells (ES cells) results in an easily identifiable phenotype upon differentiation of targeted cells into embryoid bodies (EBs) of lymphoid and endothelial origins (Keller and Orkin reviews). Degenerate homologous probes are utilized to identify other novel, related genes which function in a common pathway, and EHR is used to ablate or modify gene function. ES cells are differentiated into cells of lymphoid and endothelial origin, and screened in a similar manner to that of Gata5 mutants. –

Please replace the paragraph beginning at page 57, line 19, with the following rewritten paragraph:

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– Disruption of a single allele of the mismatch repair gene, Msh2, in ES cells results in defective response to oxidative stress induced by low-level radiation [PNAS 1998 95(20) 11915-20]. These cells have an increased survival in response to radiation through a failure to undergo apoptosis. Related genes are obtained using EHR with degenerate probes, and gene function is ablated or modified to screen for novel family members that also have the same defective response to oxidatitve stress. This is assessed by screening for survival of cells with damaged DNA resulting from apoptotic changes. In addition, EHR is used to disrupt Msh2 in both undifferentiated or stratified keratinocytes in order to determine if mismatch repair operates through a common pathway in both cell types. –

Please replace the paragraph beginning at page 57, line 28, with the following rewritten paragraph:

C42

– F: Disruption of a single allele of the human tumor suppressor gene, Patched (Ptch), [Nature Medicine Nov. 1999 Volume 5, #11 pp. 1285-1291] results in a predisposition to basal cell carcinoma, the most prevalent form of cancer in humans, in mouse skin exposed to ultraviolet (UV) and ionizing radiation. EHR is used to disrupt Ptch and other genes in the hedgehog signaling pathway in cells, including human or mouse keratinocytes and fibroblasts. Both undifferentiated and differentiated cells are screened for changes induced by UV and ionizing radiation to determine that the phenotype of the whole organism is recapitulated. –

Please replace the paragraph beginning at page 57, line 37, and continuing to page 58, with the following rewritten paragraph:

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– Some genes require disruption of multiple alleles in order to obtain a screenable phenotype, and in these instances we utilize cells with single or multiply disrupted alleles to perform mutagenesis using exact and/or degenerate EHR probes to determine other key players on a common pathway. EHR is used to disrupt a single key component in the DNA damage response pathway. Rad 51A and degenerate EHR probes to common functional domains, such as the ATP binding domain, are used to functionally modify radiation repair in cells such as ES cells, keratinocytes, and fibroblasts. –

Please replace the paragraph beginning at page 58, line 6, with the following rewritten paragraph:

– Trans-dominant mutations have been shown to play a role in a large number of highly prevalent human diseases, including nevoid basal cell carcinoma syndrome (human Ptch), Alzheimer's disease (presenilin), cardiac hypertrophy (sarcomeric proteins), familial hypercholesterolemia (LDL receptor), obesity (melanocortin-4), and hereditary non-polyposis colon cancer (DNA mismatch repair genes MLH-1 and MLH-3). [Nature Genetics vol. 24 Jan 2000 pp 27-35] We use EHR to perform insertional mutagenesis to create germline trans-dominant mutations in cell lines (such as ES, fibroblasts, keratinocytes, or transformed cell lines) for a phenotype screen. EHR mutagenesis is utilized to create dominant negative mutant forms of the DNA mismatch repair genes, MLH-1 and MLH-2, by creating truncations or chimeric truncation/GFP fusion proteins. These trans-dominant mutations are expressed in cell lines (such as ES, fibroblasts, keratinocytes, or transformed cell lines), and the fluorescence tagged mutant protein is followed to determine which mutations disrupt specific cellular functions, including subcellular distribution or trafficking. –

A44

Please replace the paragraph beginning at page 59, line 1, with the following rewritten paragraph:

– A high-throughput screen of hGR translocation has distinct advantages over ~~in vitro ligand-receptor~~ binding assays because other parameters can be screened in parallel, such as the function of other receptors, targets, or other cellular processes. Indicator cells, such as HeLa cells, are transiently transfected with a plasmid encoding GFP-hGR chimeric protein and the translocation of GFP-hGR into the nucleus is visualized. –

A45

Please replace the paragraph beginning at page 59, line 7, with the following rewritten paragraph:

– EHR is used to introduce mutations into hGR to block signaling in normal and cancer cells and to screen cells with aberrant ligand-receptor translocation. The hGR gene is biovalidated by screening for drugs that enhance or revert the altered phenotype. –

A46

On page 60, immediately preceding the claims, please insert the enclosed text entitled "SEQUENCE LISTING".

Claim 46 has been added. After entry of the present amendments, Claims 1-46 are pending.

An Appendix with pending claims and with a marked-up version of the changes in the amended claims captioned **"Version with markings to show changes made"** has been attached for the Examiner's convenience. No new matter has been introduced by way of these amendments, and entry thereof is respectfully requested.

SEQUENCE RULES COMPLIANCE

The amendments adding sequence identification numbers are made in adherence with 37 C.F.R. § 1.821-1.825. This amendment is accompanied by a floppy disc containing the above named sequence, SEQUENCE ID NUMBERS 1-12 in computer readable form, and a paper copy of the sequence information. The computer readable sequence listing was prepared through use of the software program "PatentIn" provided by the PTO. The information contained in the computer readable disc is identical to that of the paper copy. This amendment contains no new matter. Applicant submits that this amendment, the accompanying computer readable sequence listing, and the paper copy thereof serve to place this application in a condition of adherence to the rules 37 C.F.R. § 1.821-1.825.

PRIORITY

This application claims priority to provisional application serial no. 60/125,536, filed on March 22, 1999 and is expressly incorporated by reference herein.

REJECTIONS UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claim 1 is indefinite because the preamble states that "the method is for cloning a target nucleic acid" but the final process step is "isolating said target nucleic acid". The claim has been amended and support for this is found in the specification on page 7, line 24-39.

Claim 1 recites limitation "said second target polynucleotide" on page 60, line 10. This has been amended to " said second targeting polynucleotide" to provide and antecedent basis for this limitation.

Claim 5 recites limitation "said cellular library" on page 60, line 26. This claim has been amended in ln 25 by deleting the word "a target" and inserting the words "cells to make a cellular library" instead so that the limitation now has an antecedent basis.

Claim 7 has been amended and the limitation "said gene products" has been deleted thus making the claim definite.

Claim 12 has been amended to delete the word "said" since the limitation did not have sufficient antecedent basis.

Claim 30 (Office action mistakenly says claim 26) recites the limitation "said altered phenotype" on page 63 (Office action mistakenly says page 61), line 3 which has insufficient antecedent basis. Claim 30 depends on claim 27 which recites this limitation. This rejection should therefore be withdrawn.

Claim 28 has been rejected for indefiniteness and Examiner states that it is unclear what this claim means.

REJECTIONS UNDER 35 U.S.C. § 102

Claims 34-45 are rejected under 35 U.S.C.§ 102(b) as being anticipated by Kowalski, U.S.P.N. 5,139,744 (Kowalski).

Kowalski teaches an automated multi-purpose laboratory workstation with capabilities for automation of a wide variety of bioanalytical procedures including sample pipetting, serial dilution, reagent additions, mixing, reaction timing and other known manual procedures. While it teaches an apparatus that can be utilized for high-throughput methods, Kowalski does not teach utilizing recombinases (EHR techniques) in identification, isolation, cloning, screening of target nucleic acids and their expression and phenotypic screening.

The current invention is directed towards using high-throughput methods for the identification, isolation, cloning, and screening of target nucleic acids utilizing enhanced homologous recombination (EHR) techniques and their expression and phenotypic screening for biological activity using robotic systems.

As the Examiner is aware, to anticipate a claim under §102 (b), the reference must teach every element of the claim (M.P.E.P.§ 2131).

Kowalski does not teach or suggest utilizing EHR techniques for identifying and isolating target sequences and hence, Kowalski does not anticipate the claimed subject matter.

Therefore, Applicants submit that Claims 34-45 are not anticipated by Kowalski under 35 U.S.C. §102(b) and respectfully request withdrawal of the rejection.

REJECTIONS UNDER 35 U.S.C. § 103

Claims 1-4, 11, 12-18 and 33 are rejected under 35 U.S.C.§103(a) as being unpatentable over Sena et al., U.S.P.N. 5,273,881 (Sena) and (Kowalski).

Kowalski has been discussed above.

Sena teaches a compositions comprising a recombinase that can be used for isolation and enrichment of target DNA that is useful for diagnostic purposes and for mapping genes or regulatory sequences in a chromosome but does not teach use of a robotic system for high-throughput use of this composition.

The current invention has been previously discussed.

As the Examiner is aware, the test for obviousness is whether the claimed invention as a whole would have been obvious at the time it was made to a person of ordinary skill in the art. A *prima facie* case of obviousness requires that there must be some suggestion or motivation, either in the references or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. See M.P.E.P. § 2142.

The Examiner states that it would be obvious to one of ordinary skill in the art to use the robotic system of Kowalski in a method of Sena with a reasonable expectation of success to result in the current invention and the motivation is that the "robotic system would have allowed processing of a large number of samples simultaneously" (emphasis added).

However, there is no motivation to combine these references since neither Kowalski nor Sena suggest the combination. Kowalski does not suggest the use of EHR techniques to isolate and identify valid drug targets and Sena does not suggest using high-throughput methods for double D-loop applications. Applicants respectfully remind the Examiner that "[t]he mere fact that references can be combined or modified does not render the resultant

combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills,* 16 USPQ2d 1430 (Fed. Cir. 1990); M.P.E.P. § 2143.01. Accordingly, the Applicants submit that there is no motivation to combine these references and submit that Claims 1-4, 11, 12-18 and 33 are not obvious over Kowalski and Sena thereby requesting withdrawal of the rejection.

Claims 5-6, 19-20, 25-27, 29-32 are rejected under 35 U.S.C.§103(a) as being unpatentable over (Sena) and (Kowalski) as applied to claims 1 and 12 and further in view of Short, U.S.P.N. 6,057,103 (Short).

Sena and Kowalski have been discussed before.

Short teaches generation of gene expression libraries in cells and screening of these cellular libraries. Short does not teach use of robotic systems.

The invention taught by the above claims is directed towards generating target DNA libraries by EHR techniques; isolation, cloning and screening the DNA libraries; and introducing the isolated DNA into cells and screening for bioactive agents; wherein all steps use robotic systems.

The Examiner states that it would be obvious to one of ordinary skill in the art to combine Short, Sena and Kowalski with a reasonable expectation of success, stating that, the motivation to do so is that integrated genomics required screening of a large number of libraries and cells against bioactive agents and the process " would have been made more efficient and economical by using a robotic system" (emphasis added).

However, as mentioned earlier, there is no motivation to combine these references since neither Kowalski nor Sena nor Short suggest the combination. Neither Kowalski nor Short suggest the use of EHR techniques for isolating and identifying target nucleic acids. Sena does not suggest using high-throughput methods for double D-loop applications nor the generation of libraries for screening drugs. Even though Short teaches screening of large number of libraries and cells against bioactive agents, it does not teach or suggest the use of a robotic system for efficiency and economy of screening. Applicants respectfully remind the Examiner that "[t]he mere fact that references can be combined or modified does not render

the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills,* 16 USPQ2d 1430 (Fed. Cir. 1990); M.P.E.P. § 2143.01. Accordingly, the Applicants submit that there is no motivation to combine these references.

Claims 7-10 and 21-24 are rejected under 35 U.S.C.§103(a) as being unpatentable over (Sena) and (Kowalski) as applied to claim 12 and further in view of Ghai et al., U.S.P.N. 5,955,269 (Ghai).

Sena and Kowalski have been discussed before.

Ghai teaches methods of screening for the presence of bioactive substances in food that can modulate the expression of one or more genes that are related to or associated with disease. Ghai also teaches the use of laboratory robots for transfers and other manipulations. But Ghai does not teach the use of EHR techniques to isolate and enrich nucleic acid targets that can be cloned into cells to prepare libraries for use in screening for drug targets.

The current invention is directed towards using high-throughput methods using robotic systems for the identification, isolation, cloning of nucleic acids utilizing enhanced homologous recombination (EHR) techniques and phenotypic screening of target nucleic acids.

The Examiner states that it would be obvious to one of ordinary skill in the art to combine Ghai and Sena with a reasonable expectation of success. The motivation is that integrated genomics required screening of large number of libraries and cells against bioactive agents and the process " would have been made more efficient and economical by using a robotic system" (emphasis added).

However, there is no motivation to combine these references since neither Sena, Kowalski nor Ghai suggest the combination. Kowalski and Ghai do not suggest the use of EHR techniques to isolate and identify valid drug targets and Sena does not suggest using high-throughput methods for double D-loop applications. Again, Applicants respectfully remind the Examiner that "[t]he mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills,* 16 USPQ2d 1430 (Fed. Cir. 1990); M.P.E.P. § 2143.01. Accordingly, Applicants submit that there is no motivation to combine the references.

CONCLUSION

Applicants respectfully submit that the claims are now in condition for allowance and early notification to that effect is requested. If the Examiner feels there are further unresolved issues, the Examiner is requested to phone the undersigned at (415) 781-1989.

Respectfully submitted,

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Date: 5/24/01

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